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New Cellulose Derivatives for the Isolation of Biologically Active Molecules

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The search for the relationship between structure and function of antibodies has gone through different phases. Considerable data has been accumulated concerning the interactions of antibody with antigens, particularly with respect to haptens of related and different structures. These data have given rise to a multitude of speculations concerning the chemical and physical structure of antibodies and the mechanism of antibody reactions. At present, major questions awaiting answers are (1) Does antibody to a particular antigen have a unique primary structure?, (2) Do all antibodies have the same primary structure but they are folded differently?, (3) Are the antibody combining sites for a particular antigen the same, but are other parts of the antibody molecule modified for other functions?, (4) What is the composition and structure of the active site?

The evidence that antibody produced against protein antigens is heterogeneous, and the possibility that antibody to haptens is heterogeneous makes it mandatory that any studies concerning antibody structure or interaction be made on <u>all</u> the antibody from a selected quantity of serum, unless it can be shown that a representative sample has been obtained during the isolation process. Conclusions based on comparisons of peptide or amino acid analyses, and other antibody components are particularly sensitive to fractionation of antibody during its isolation from serum.

For these reasons, we have concentrated our present efforts on investigating methods for isolating the maximum possible quantity of antibody from a serum sample, in as pure a form as possible, measured

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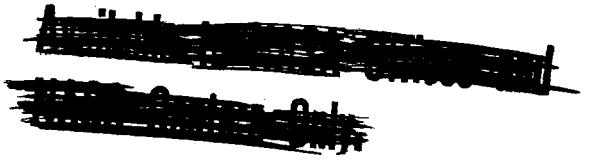
by the fraction precipitable with specific antigen. Available methods have drawbacks which include incomplete separation from antigen, low overall yield, low purity, lack of generality and very involved procedures. The most promising approach is that of chemically coupling the antigen to an insoluble derivative of a polymeric carrier such as an aromatic or aliphatic resin, a protein or a cellulose derivative. In principle, contamination of antibody by antigen should be negligible and separation from other serum proteins should be complete, if conditions are properly controlled. A review of this and other isolation techniques has recently been published[1].

The most frequently used insoluble antigen is polyaminopolystyrene, which has been diazotized and coupled to protein antigens[2]. This derivative has the disadvantage that recovery of antibody is low, overall yields are low and appreciable amounts of nonspecific protein are released with the antibody[1]. In addition, the coupling reaction is limited to substances which will couple to aryldiazonium compounds. To extend the scope and utility of this technique we developed the use of dicyclohexylcarbodiimide as a coupling agent for chemically binding antigenic substances with basic amino groups, to insoluble varieties of carboxymethylcellulose[3]. Water soluble diimides such as 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide can also be used. Recovery of antibody complexed to the cellulose derivatives was in excess of 88%. Since the original work was done, overall yields exceeding 90% are now being consistently obtained from anti-BSA serum and anti-azophenylarsonate serum, by dissociating the antigen-antibody complex with 1% saline acidified with hydrochloric acid to pH 2.3 for the former case, and to pH 2.0 for the latter. Human gamma globulins,



bovine serum albumin, keyhole limpet hemocyanin and other proteins can be coupled. Many enzymes couple and still retain activity. In addition, p-(p'-aminophenylazophenyl)-arsonic acid, ε-DNP-lysine and other organic amines reacted with carboxymethylcellulose and could be used to bind serum antibody. Coupling of the amines to carboxymethylcellulose was more effective at pH 3.6-4.0 than near neutral pH.

Carboxymethylcellulose coupled to antigens releases about 0.01 mg of nonspecific protein per ml of serum per g of carboxymethylcellulose. Another cellulose derivative commercially available (Cellex PAB) has been bound to proteins by diazotization and coupling[4]. Any uncoupled diazo groups remaining were blocked by reaction with β-naphthol. While adequate for the isolation of antibody, it is a deep brown substance which is difficult to free of water soluble material. The coupled Cellex PAB retains about 0.1 mg of nonspecific protein per ml of serum per g of Cellex PAB[3].

Coupling through azo links is useful and sometimes advantageous. We have now developed a simple, rapid method of synthesizing a new aryl-aminocellulose under mild conditions using a slight modification of the method we use for coupling proteins and haptens directly to carboxymethyl-cellulose (CMC)[3]. The product is a clean white solid which can be diazo-tized, or used as is with dicyclohexylcarbodiimide for coupling to various organic and biological substances.

Benzidine, first suspended in water, was coupled to ten times its weight of carboxymethylcellulose by adding a tetrahydrofuran solution of dicyclohexylcarbodiimide (DCC). The slurry was allowed to stand for two days at room temperature, after which the coupled material was washed

exhaustively with acetone, dilute acid, base and finally water.

The number of benzidine groups coupled to the cellulose was determined by first reacting the benzidine derivative with a solution of benzenesulfonyl chloride in tetrahydrofuran (in the presence of triethylamine) to form sulfonamides with the free amino groups. After hydrolyzing any anhydride that formed, the remaining free carboxyl groups were titrated with sodium hydroxide. Typical data were: 1.0 g carboxymethylcellulose of 0.78 meq free carboxyl groups per gram was reacted with 0.1 g of benzidine to form a derivative with 0.2 meq of free carboxyl groups per gram. By difference, the benzidine coupled amounted to 0.58 meq/g or 0.1 g, signifying that all the benzidine reacted with the cellulose.

An immunoadsorbent was prepared by diazotizing the arylamino-cellulose and coupling it to bovine serum albumin. Subsequent treatment with β-naphthol blocked any unreacted diazonium groups. One gram of this material was poured into a 10 mm glass tube, with a glass wool plug, to make a column several centimeters m long.

Five ml of rabbit anti-bovine serum albumin was passed through the column followed by 1% saline until no protein could be detected using a 1 mm flow-through cell in a spectrophotometer set at 220 mμ. Complexed material was eluted from the column with 1% NaCl acidified to pH 2.3 with hydrochloric acid. All the specific antibody contained in the serum was picked up and subsequently released. The isolated antibody was not less than 80% precipitable with specific antigen (Table 1).

Similar experiments were performed using a nonspecific rabbit

serum. In Table 2 the quantity of nonspecific protein eluted from an immunoadsorbent made from a carboxymethylcellulose derivative with 0.52 meq/g free amino groups is compared with that eluted from an immunoadsorbent made from a p-aminobenzyl derivative with 0.32 meq/g free amino groups.

Under the conditions used, the retention and release of non-apecific protein on these immunoadsorbents appears to be dependent on the number of β-naphthyl groups present. Carboxymethylcellulose which is directly coupled to an antigen through an amide linkage and requires no blocking groups, retains no more than 0.011 mg for each ml of serum passed through a one gram column[3]. A p-aminobenzyl immunoadsorbent retains about 0.1 mg nonspecific protein while the new arylaminocellulose, although still useful as an immunoadsorbent, has about twice the number of reactive groups as the p-aminobenzylcellulose and retains about twice the quantity of non-specific protein. By reducing the number of benzidine groups, nonspecific protein adsorption should be correspondingly reduced. Such immunoadsorbents are useful because they contain proteins coupled at reactive sites other than those involved in the reaction with dicyclohexylcarbodiimide.

We are presently exploring the coupling of this new derivative to other proteins and organic molecules both by diazotization and by amide formation. By chemically coupling inhibitors or other complexing agents to the cellulose derivatives we expect that biologically active molecules and other substances can be concentrated and isolated.

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Table 1.

Isolation of Antibody from a One Gram Column of Carboxymethylcellulose Benzidine Derivative Coupled to Bovine Serum Albumin.

Serum Added (ml)	Total Antibody Passed Through Column (mg)	Total Protein Released at pH 2.3 (mg)	Percent Precipitable Protein
5	10.50	11.25	82.1
5	6.30	6.78	80.5

Table 2.

Comparison of Carboxymethylcellulose Benzidine Derivative and p-Aminobenzylcellulose Derivative for Release of Nonspecific Protein at pH 2.3 From 1g Columns

		Protein Eluted	
Column & Antigen	Serum added (ml)	per ml	Total (mg)
PAB-BSA	2	0.12	0.24
PAB-BSA	3	0.08	0.24
CMCB-BSA	5	0.18	0.90
CMCB-BSA	5	0.16	0.77

PAB - p-Aminobenzylcellulose

CMCB - Carboxymethylcellulose coupled to benzidine

BSA - Bovine serum albumin

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